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14. ABSTRACT

The goal of these studies is to develop and validate cell-penetrating bi-specific antibodies as an agent that can selectively inhibit the function of intracellular proteins. We have developed 3E10-AR441 bi-specific antibody to inhibit the function both ligand-dependent and independent forms of the androgen receptor (AR), key drivers of prostate carcinogenesis. Key accomplishments in this reporting period are the production of a prototype 3E10-AR441 bi-specific antibody, and demonstration of target engagement under denaturing and non-denaturing conditions, as well as in intact cell systems. The antibody inhibited the growth of prostate cancer cells. We will continue to characterize the biochemical lesions produced by this antibody, while developing higher affinity analogs with improved production characteristics.

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INTRODUCTION

This report covers research activities supported by DOD contract W81XWH-12-1-0534, entitled Cell-penetrating bispecific antibodies for targeting oncogenic transcription factors in advanced prostate cancer. The research is a collaborative effort between Michael Lilly, MD (Principal Investigator) and Richard Weisbart (Co-investigator). Dr. Lilly is based at the Hollings Cancer Center, Medical University of South Carolina, while Dr. Weisbart is at the Sepulveda VA, affiliated with the University of California Los Angeles. This contract was activated at MUSC on October 1, 2012. The reporting period is therefore from 10/1/2012 through 9/30/2013. Work under this contract began in Dr. Lilly's laboratory about November 1, 2012, with the recruitment of Nancy Goicochea-Pa, PhD to a postdoctoral position. Dr. Weisbart's group has worked from December, 2012, following the completion of a subcontract agreement between MUSC and Dr. Weisbart's institution.

Three specific aims were proposed. In the first, Dr. Weisbart's group would produce a prototype bispecific antibody targeting the androgen receptor (AR) and optimize its structure and production. The second specific aim, to be carried out by Dr. Lilly's laboratory, focuses on the biochemical and biologic properties of the bispecific antibody, through the use of biochemical and biologic assay systems. The final specific aim will examine the ability of the bispecific antibody to perturb the growth of prostate cancer cells in murine models. Additional studies will characterize PK/PD parameters of the antibody. Substantial progress has been made in the first two aims. Work has not yet started on the third aim.

BODY

1. Weisbart Group

A. Production of 3E10-AR441 bispecific antibody (Fig. 1). During the past year we have made major progress in accomplishing the goal of producing a bispecific single-chain (sc) Fv fragment consisting of the Fv fragment of mAb 3E10, an intracellular transporter and mAb AR441, an antibody that binds the androgen receptor. The molecular fusion protein was constructed and shown to be correct by sequencing and characterized by SDS-PAGE. As shown in Fig. 1, the fusion protein is twice the size of the 3E10 Fv alone as expected. An SDS gel shown is stained with Gel-Code Blue.

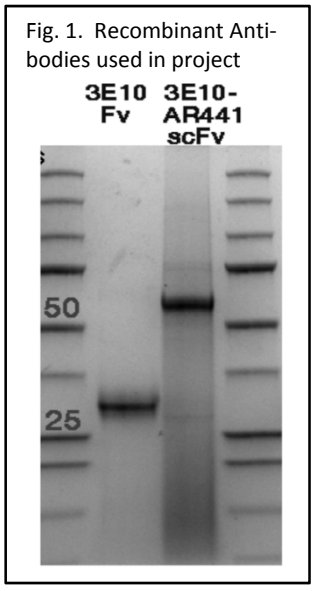


Fig. 1. Recombinant Anti-bodies used in project

We have produced a moderate amount of the bispecific antibody, both for our use and for Dr. Lilly's laboratory. We have supplied approximately 20mg of material to Dr. Lilly over the past year. The production of this protein remains inefficient. We are now exploring the effects of modifications to the linker structures and the arrangement of the various protein domains on efficiency of expression.

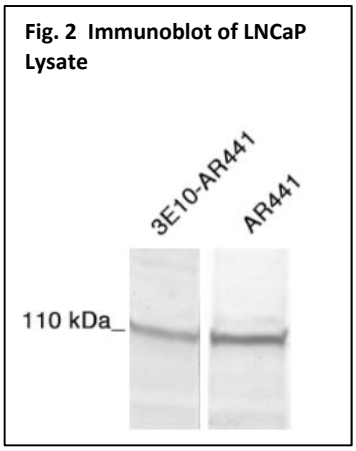


Fig. 2 Immunoblot of LNCaP Lysate

B. Binding of bispecific antibody to denatured target protein (Fig. 2). In order to determine if 3E10-AR441 bispecific sc Fv, was able to bind the androgen receptor. A western blot was done with lysate from LNCaP cells containing the androgen receptor. As shown in Fig. 2, 3E10-AR441 bound the 110kd androgen receptor as shown by comparison with reactivity of AR441 hybridoma monoclonal antibody.

2. Lilly Group

A. Subcellular Localization of bispecific antibody (Fig. 3). LNCaP cells were incubated with the scFv 3E10 antibody, the bispecific 3E10-AR441 antibody or control medium containing FCS, x 18hr. The cells were then fixed with MeOH, and stained with secondary reagent (anti-MYC-FITC

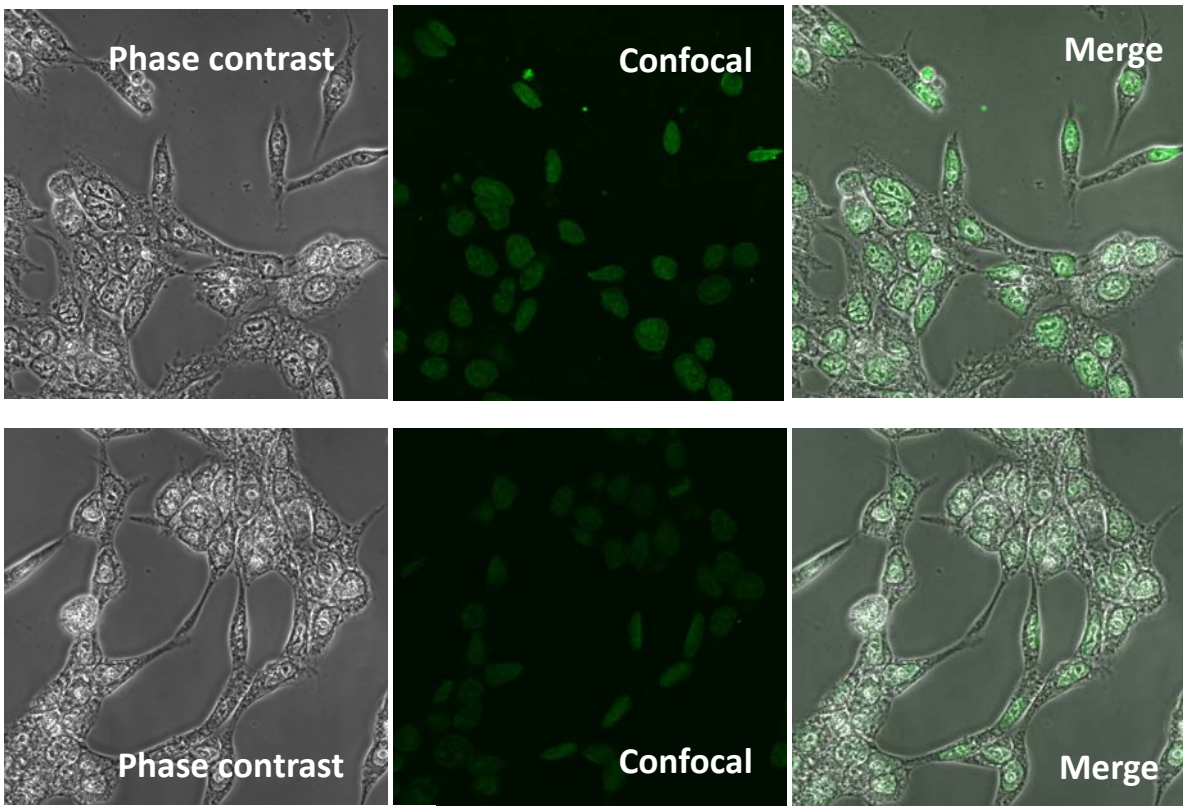
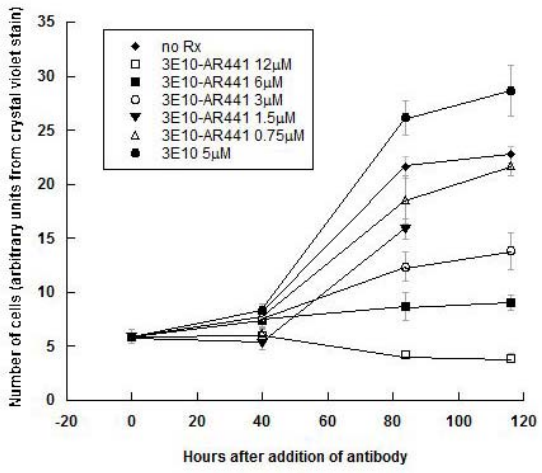


Fig. 3. Confocal microscopy of LNCaP cells treated with ScFv 3E10 or with bispecific antibody 3E10-AR441

conjugate). The cells were then examined by confocal microscopy. There was no staining of the cells initially labelled with medium alone (not shown). However, cells treated with either scFv 3E10, or with the 3E10-AR441 bispecific antibody had definite nuclear staining (Fig. 3). The concentration of the bispecific was about half that of the scFv, leading to paler staining. These data demonstrate that the scFv 3E10 and its bioconjugates can penetrate prostate cancer cells and localize in the nucleus.

Fig. 4 Inhibition of growth of 22Rv1 cells by 3E10-AR441 bispecific AB

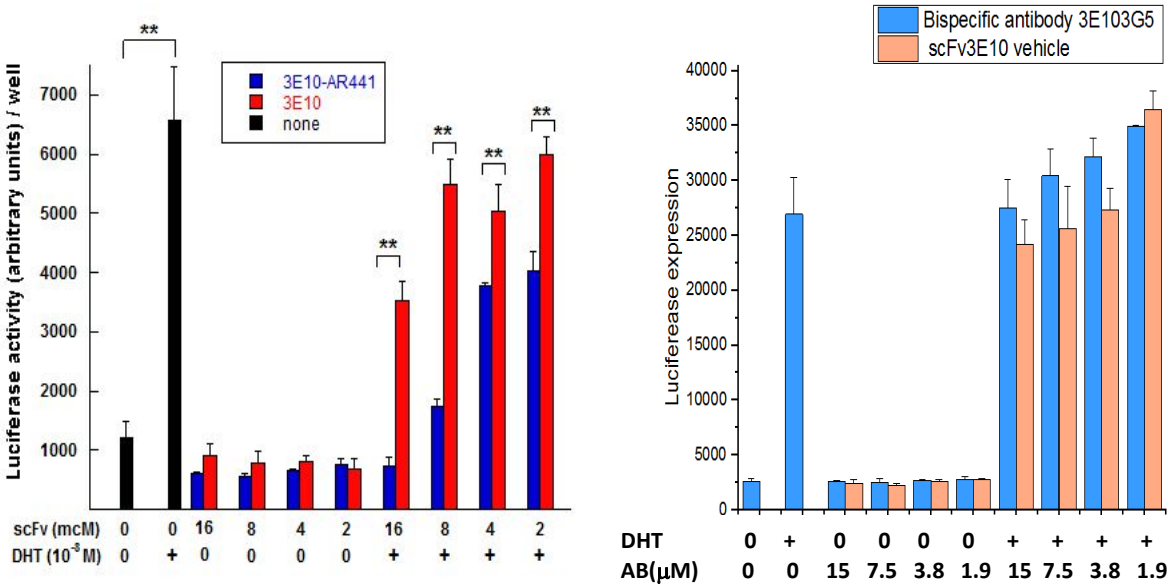


B. Growth inhibition by Bispecific AB. 22Rv1 prostate cancer cells were plated in 96well flat-bottom plates and then incubated with varying amounts of 3E10-AR441 bispecific antibody.

Growth continued for 120hrs, then the cells were fixed and stained with crystal violet. The bispecific 3E10-AR441 antibody inhibited growth in a dose-dependent manner (Fig. 4). We are looking at the growth effects of 3E10-AR441 bispecific antibody on a wide variety of prostate and other tumor cells.

C. Inhibition of AR genomic signaling by bispecific antibody (Fig. 5). A key goal of specific aim #2 was to demonstrate that the 3E10-AR441 antibody can engage its target (AR) and interrupt AR-dependent signaling and effects. The proposed assay system required the engineering of BPH1 cells with WT or mutant ARs. These cells would then be treated with 3E10-AR441, with a growth-dependent readout to document an effect of AR signaling. This has proved to be impractical. We have repeatedly failed to establish stable AR transfectants in BPH1 cells. We have therefore used an alternative system to demonstrate target engagement and its functional consequences.

Fig. 5. Inhibition of androgen-stimulated, AR-mediated gene expression. *Left panel:* comparison of 3E10 vs 3E10-AR441. *Right panel:* comparison of 3E10 vs 3E10-3G5

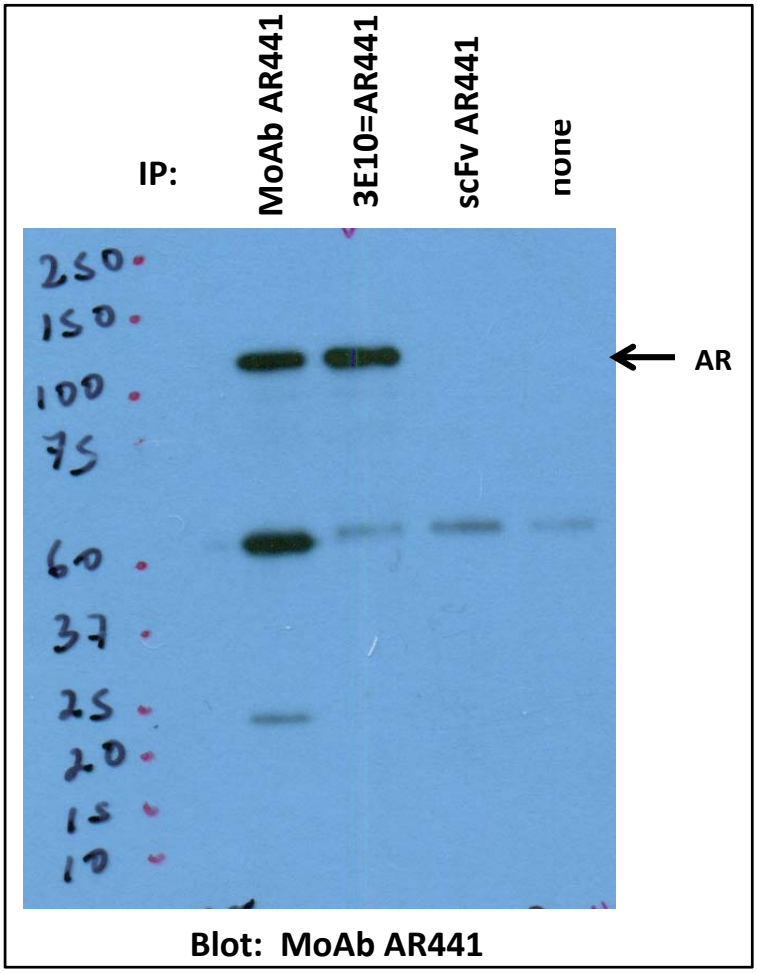


LNCaP cells were successfully engineered to express a firefly luciferase gene under the control of a promoter containing multiple tandem copies of a consensus androgen response element (ARE; LNCaP/luc cells). The LNCaP/luc cells are routinely grown in RPMI1640 medium with FCS 10%. For assay of androgen-dependent signaling through the AR, the cells are transferred to RPMI 1640 medium containing an androgen-free serum substitute at 5% (KO Serum; Invitrogen). After culture for 18-24hrs in a low-androgen environment, the cells are pulsed with dihydrotestosterone (DHT) x 6-8hrs. They are then lysed and assayed for firefly luciferase content using a commercial kit (Promega). Luciferase activity is normalized to the amount of lysate protein. In this system dihydrotestosterone at 10nM routinely produces a 6-10-fold increase in luciferase content.

To determine if 3E10-AR441 bispecific antibody can disrupt signaling through the AR, we cultured serum-starved LNCaP/Luc with 3E10-AR441, scFv 3E10 alone, or another bispecific antibody 3E10-3G5 targeting the MDM2 protein. Neither scFv 3E10 or the 3E10-3G5 anti-MDM2 bispecific antibody had any effect of AR-dependent transcription of luciferase (Fig. 5 right panel). In contrast the 3E10-AR441 bispecific antibody substantially abrogated DHT-induced luciferase expression in a dose-dependent manner (Fig. 5, left panel). Nearly complete blockade of AR signaling occurred

with antibody concentrations of 8-16mcM. These data indicate that the bispecific antibody can enter prostate cancer cells and interrupt AR function.

D. **Direct binding of bispecific antibody to its molecular target, AR** (Fig. 6). To demonstrate directly that 3E10-AR441 can bind to WT and mutant ARs, we have examined the ability of 3E10-AR441 to bind to AR in native conformation in an immunoprecipitation format. Test antibodies were mixed with a lysate of LNCaP and other prostate cancer cells as a source for AR. The lysate was prepared with non-denaturing detergents. Immune complexes were recovered with Protein L-agarose, which binds to conserved domains of K light chains found both in native antibodies and most scFv antibodies. The complexes were then recovered from the solid phase with SDS, and detected by immunoblotting, using anti-AR and other antibodies.



The parental MoAb AR441 and the bispecific 3E10-AR441 antibodies were able to bind to both wild type AR (LNCaP cells; Fig 6) and ligand-binding-domain-deficient splice variants reported from 22Rv1 and VCaP cells (not shown). Interestingly the scFv AR441 alone was unable to bind to the native conformation of AR in a precipitation assay. This suggests that the whole bispecific antibody develops specific conformations that are not present in the separate component scFvs.

E. **Binding affinity of parental MoAb AR441 and bispecific antibody 3E10-AR441**. To estimate the binding affinity of 3E10-AR441 compared with the parental MoAb AR441, we used a variety of biochemical assays. Our initial attempts used a flow cytometry assay to measure the binding of AR441 to fixed, permeabilized LNCaP cells. The later served as a convenient form of native WT (and T877 mutant) AR on a solid phase. A FITC-labelled AR441 antibody will bind to these cells, and the binding can easily be quantified by flow cytometry. By using unlabeled AR441 to compete with the FITC-labelled molecule, we can calculate the binding affinity (2). Our estimate is that the Ki is 23nM, consistent with values expected for a monoclonal antibody. However, when we attempted to use the scFv 3E10 or 3E10-AR441 for competition with the FITC-labelled AR441 we found high levels of non-specific binding of the 3E10 antibody or its conjugates. We are now exploring alternative approaches using a sandwich ELISA format. We have not obtained quantitative information yet, but it appears that the binding affinity of 3E10-AR441 to native WT AR is somewhat less than that of the parental MoAb AR441. This suggests that some effort may be needed through directed molecular evolution techniques to enhance the affinity of the scFv AR441 hemi-antibody.

F. Development of cell lines stably expressing WT and mutant ARs. To most precisely catalogue the AR forms that react with 3E10-AR441 we will still need to express these individually in mammalian cells that lack substantial amounts of endogenous AR. Since BPH1 cells were not satisfactory we are now preparing stable transfectants of HEK cells for this purpose. HEK/AR WT and HEK/AR Q640X cell lines have already been prepared. Other cell lines containing mutant ARs are under development. We will transduce these cell lines to express the ARE-driven luciferase gene, allowing us to have only 1 form of AR in each cell line, for both immunologic study and functional analysis. We are also making these into LNCaP cells as a backup plan. LNCaP already have a WT and T877 mutant AR alleles, but are relatively easy to transduce. They may allow us to obtain some data if the HEK cell lines cannot be used to reconstitute a DHT→AR→luciferase signaling axis.

KEY RESEARCH ACCOMPLISHMENTS

1. Weisbart Group
   A. Design, Synthesis, and Production (>10mg) of the 3E10-AR441 bispecific antibody
   B. Production of control scFv and bispecific antibodies scFv3E10, 3E10-3G5, and scFvAR441
   C. Demonstration of binding of the scFv AR441 antibody to AR in an immunoblotting system

2. Lilly Group
   A. Demonstration of nuclear localization of 3E10-AR441 and scFv 3E10 in the nucleus of LNCaP cells by confocal microscopy
   B. Growth inhibition experiments using a variety of prostate cancer cell lines
   C. Demonstration that 3E10-AR441 inhibits androgen-dependent signaling through the AR, using LNCaP cells engineered to expess firefly luciferase under the control of a synthetic ARE. scFv3E10, and another bispecific antibody targeting MDM2, 3E10-3G5, do not inhibit AR signaling.
   D. Demonstration that 3E10-AR441 can bind to wild-type and LBD-deficient splice variants of the AR under “native” conditions, using an “immunoprecipitation” assay with a protein L-coated solid phase
   E. Measurement of the binding affinity of the parental AR441 monoclonal antibody to AR, and development of methods to extend these studies to 3E10-AR441.
   F. Development and characterization of test cell lines for study of 3E10-AR441:
      i) LNCaP-ARELuciferase
      ii) LNCaP/WT AR
      iii) LNCaP/AR Q640X
      iv) HEK/WT AR
      v) HEK/AR Q640X

REPORTABLE OUTCOMES

1. The 3E10-AR441 bispecific antibody will enter prostate cancer cells and localize predominately in the nucleus
2. The 3E10-AR441 bispecific antibody can bind to a variety of AR forms, including receptors lacking the ligand-binding domain, under non-denaturing conditions.
3. The 3E10-AR441 bispecific antibody inhibits genomic signaling by the wild type (and presumably T877 mutant) AR forms in LNCaP cells.

CONCLUSIONS

1. The bispecific 3E10-AR441 antibody has performed as expected in tissue culture experiments, by entering cells, translocating to the nucleus, and blocking androgen-dependent signaling through the AR. The antibody binds to both wild-type and LBD-mutant ARs, demonstrating robust target engagement.
2. The binding affinity of the 3E10-AR441 is probably several-fold lower than that of the parental monoclonal AR441. This may require much larger amounts of antibody than expected for a significant *in vivo* biologic effect.
3. Further protein engineering of the 3E10-AR441 antibody is probably needed to 1) enhance the binding affinity to the target protein, and 2) to improve yields from the current yeast expression system
4. The studies accomplished to date by both the Lilly and Weisbart laboratories represent a substantial completion of the propose studies for months 1-12 under specific aims #1, 2, as outlined in the approved Statement of Work.

REFERENCES

none

APPENDICES

none